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14. ABSTRACT Heterotopic ossification (HO) is the abnormal formation of bone in soft tissues and is a frequent complication in patients who have suffered traumatic brain and spinal cord injuries. Currently there are few effective treatments for this condition. This research seeks to develop engineered osteoclasts as a local cell therapy for the prevention and/or regression of HO. The goals of this research are to develop proof-of-principle data in excised human HO specimens and in an animal model using existing murine engineered osteoclasts and to develop an off-the-shelf human induced pluripotent stem (iPS) cell source for clinical translation of this technology. To date we have performed studies to optimize various hydrogel formations as cell delivery vehicles. We will use these hydrogels to retain engineered osteoclasts at sites of HO in vivo. We have also created and validated a lentiviral vector containing an inducible human RANK fusion gene that will be used to develop our human iPS cell source.					
15. SUBJECT TERMS Heterotopic ossification, osteoclast, RANK, chemical inducer of dimerization, human induced pluripotent stem (iPS) cell					
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1. **INTRODUCTION:** Heterotopic ossification (HO) is the abnormal formation of bone in soft tissues and is a frequent complication in patients who have suffered traumatic brain and spinal cord injuries. Currently, there are few effective treatments for this condition. This research seeks to develop engineered osteoclasts as a local cell therapy for the prevention and/or regression of HO. The goals of this research are to develop proof-of-principle data in excised human HO specimens and in an animal model using existing murine engineered osteoclasts and to develop an off-the-shelf human induced pluripotent stem (iPS) cell source for clinical translation of this technology.
2. **KEYWORDS:** Heterotopic ossification, osteoclast, RANK, chemical inducer of dimerization, human induced pluripotent stem (iPS) cell.
3. **OVERALL PROJECT SUMMARY:**

Current Objectives:

Major Task 1: To determine the ability of murine engineered osteoclasts to prevent or regress HO *in vivo* using a nude mouse model of traumatic HO generated by BMP injection in traumatized muscle.

- Subtask 1: Generation of RAW-iRANK cells for testing *in vivo* (PI: Giachelli, UW)
- Subtask 2: Optimization of local cell delivery at time of surgical implantation and after HO has formed in mice (PI: Sangeorzan, Harborview)

Major Task 2: Test the ability of murine engineered osteoclasts to resorb calcified deposits in human HO samples derived from traumatic bone injuries.

- Subtask 1: Collection of human HO samples (PI: Sangeorzan, Harborview)

Major Task 3: To develop human iPS cells as a cell source for engineered osteoclasts.

- Subtask 1: Lentiviral construct, lentiviral production, and transduction of human iPS cells (PI: Giachelli, UW)

Results, Progress and Accomplishments with Discussion:

SOW Major Task 1: To determine the ability of murine engineered osteoclasts to prevent or regress HO *in vivo* using a nude mouse model of traumatic HO generated by BMP injection in traumatized muscle.

Subtask 1 & 2: Generation of RAW iRANK cells for testing *in vivo* and optimization of local cell delivery at time of surgical implantation and after HO has formed in mice

Confirmation of HO formation in nude mice: (PI: Sangeorzan, Harborview) For our *in vivo* studies we are using nude mice to allow for the transplantation of allogeneic engineered cells. Prior to initiating cell delivery studies, we performed a pilot study to confirm that the absence of lymphocytes in nude mice does not

affect HO formation. For this pilot study, nude mice were anesthetized and Matrigel carrier impregnated with BMP4 (2.5 ug) was implanted in the mid-belly of the right calf. HO formation was evaluated by microCT at 10, 17, and 28 days post-implantation and the volume of ectopic bone nodules and the percent mineralization was calculated. As expected, ectopic bone nodules formed in nude mice following implantation with BMP4/Matrigel. As shown in Figure 1, nodule size increased with time. At Day 28 the average nodule volume was 1 mm³ and the percent mineralization (BV/TV) was 24.7%.

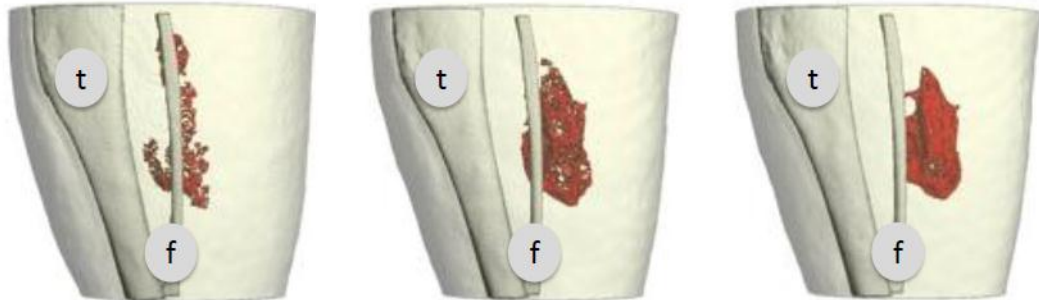


Figure 1. Serial microCT images of mineralizing ectopic bone at 10, 17 and 28 days following injection of BMP4-Matrigel. The mineralizing bone (orange) is located in the midbelly of the calf muscle group, posterior of the tibia (t) and medial to the fibula (f).

In vitro evaluation of cell delivery vehicles: (PI: Giachelli, UW) We initially proposed using collagen hydrogels as cell delivery vehicles to deliver engineered cells to sites of HO formation *in vivo*. During the past year we have performed *in vitro* studies to determine whether encapsulating engineered RAW iRANK cells in hydrogels affects osteoclastogenesis. In initial studies, engineered RAW iRANK cells were encapsulated in varying concentrations of collagen and were cultured in the presence of AP20187 to induce osteoclastogenesis. To encapsulate cells in collagen gels, cells were resuspended in media supplemented with neutralizing buffer (100 mM HEPES in 2x PBS). Collagen was then added and mixed quickly before plating gels in 4-well chamber slides. As a control, cells were plated directly on the surface of chamber slides. Media containing 50 nM AP20187 was added to the top of the gels and cells were cultured for 7 days. Cells were then stained for TRAP and the number of osteoclasts per high powered field (HPF) was counted via microscopy. Encapsulating cells in collagen gels had no significant effect on osteoclast formation compared to control cells plated directly on culture dishes (Figure 2).

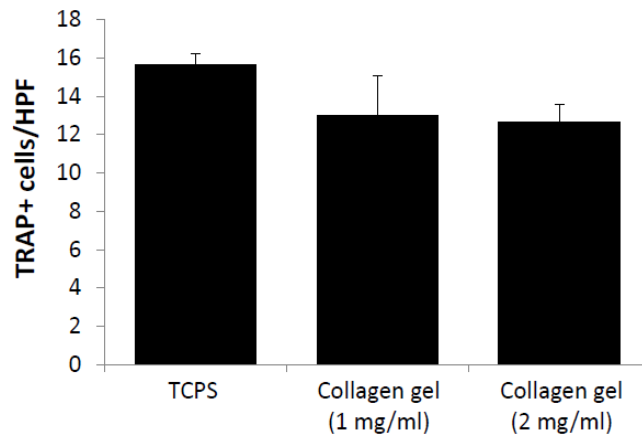


Figure 2. Encapsulation of engineered cells in collagen gels does not significantly affect osteoclastogenesis. Engineered cells were encapsulated in varying concentrations of collagen gels and osteoclastogenesis was induced by CID treatment. As a control, cells were plated directly on culture dishes (TCPS).

We subsequently tested two additional hydrogel formulations (fibrin and Matrigel) as potential carriers for *in vivo* cell delivery. For these experiments, engineered RAW iRANK cells were encapsulated in collagen, fibrin, or Matrigel at 0.5 mg/ml. Media containing 50 nM AP20187 was added to the top of the gels and cells were cultured for 7 days. Cells were then stained for TRAP and the number of osteoclasts per well was quantified via microscopy. The number of osteoclasts formed in each type of gel was not significantly different (Figure 3).

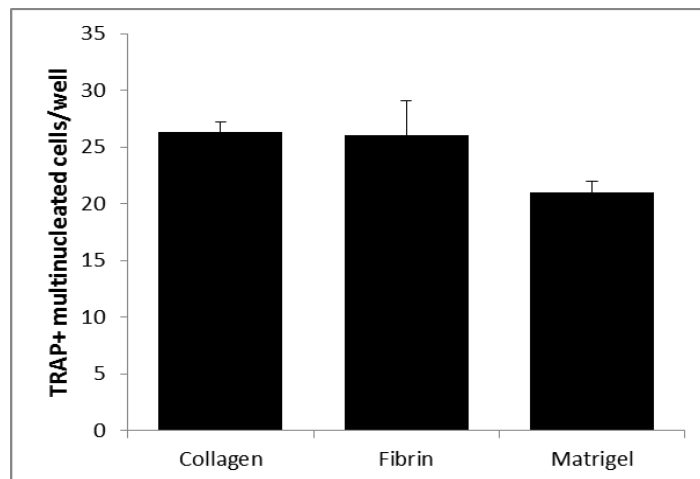


Figure 3. Encapsulation of engineered iRANK cells in hydrogels does not significantly affect osteoclastogenesis. Engineered cells were encapsulated

in collagen, fibrin, or matrigel and osteoclast formation was induced by CID treatment.

Optimization of local cell delivery at time of surgical implantation and after HO formed in mice: (PI: Sangeorzan, Site: UW) After determining that encapsulation of engineered cells does not affect osteoclastogenesis *in vitro*, we proceeded to test our hydrogel carriers for their ability to retain engineered RAW iRANK cells at sites of HO formation *in vivo*. For this study, nude mice were implanted with BMP-Matrigel in the mid-belly of the right calf to induce heterotopic bone formation. Following the BMP-Matrigel injection, mice were allowed to ambulate freely for 4 weeks. After 4 weeks, mice were injected with RAW iRANK cells expressing a luciferase reporter directly at the site of HO formation. Cells were delivered via three different hydrogels; collagen, fibrin, and Matrigel. A no gel control was also included. To induce osteoclast formation of RAW iRANK cells, mice were treated with the CID drug, AP20187. AP20187 (2 mg/kg, intraperitoneal) was administered daily for the first 4 days and every other day for the remainder of the study.

Cell retention at sites of HO formation was assessed using a Xenogen In Vivo Imaging system and mice were imaged at Days 2, 3, 4, 8, 15 after cell delivery. As shown in Figure 4, cells were retained at sites of injection in all gel formulations and in the no gel control as well. However, differences in the cell distribution were noted with the different gel carriers. The Matrigel carrier resulted in a diffuse distribution of cells in the calf, while the fibrin hydrogel carrier had the tightest distribution. However, the fibrin gel polymerized rapidly resulting in technical difficulty in administration of the gel.

Results from the Xenogen imaging were quantified as shown in Figure 5. The number of cells at the site of injection increased with time for all delivery vehicles. Interestingly, the no gel control and the collagen hydrogel had the greatest cell numbers at the injection site at Day 15.

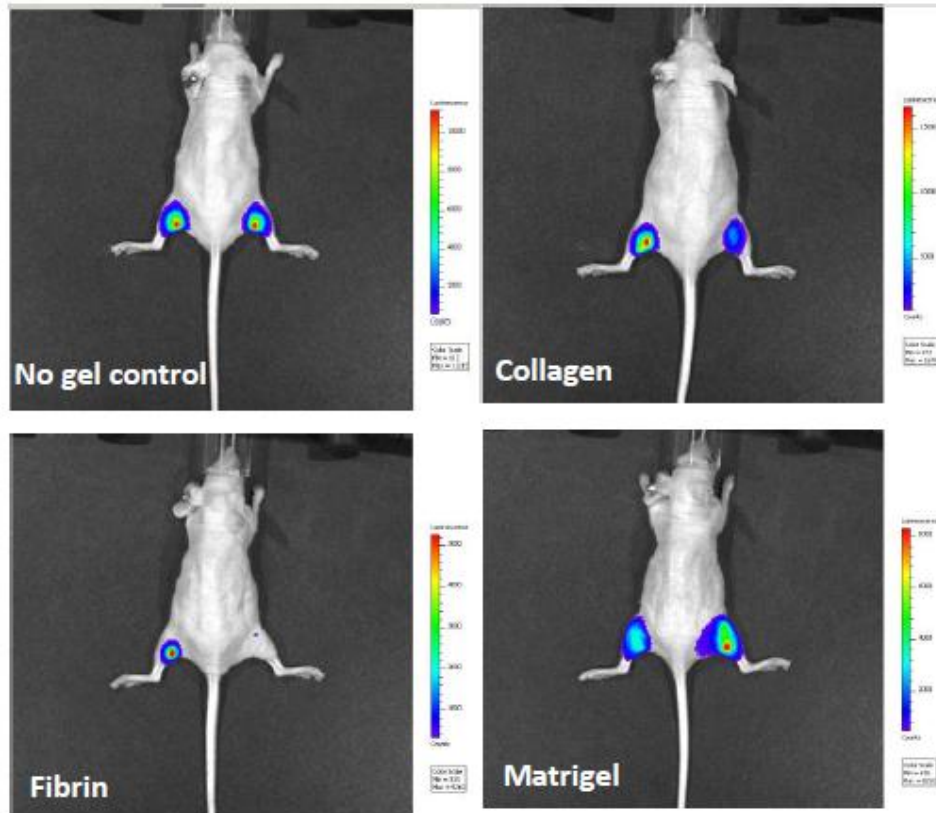


Figure 4. Hydrogel delivery vehicles efficiently retain RAW iRANK cells at the site of injection. RAW iRANK cells expressing a luciferase reporter were injected into the midbelly of the calf. Cell retention was monitored using Xenogen imaging. Representative images from each group at Day 8.

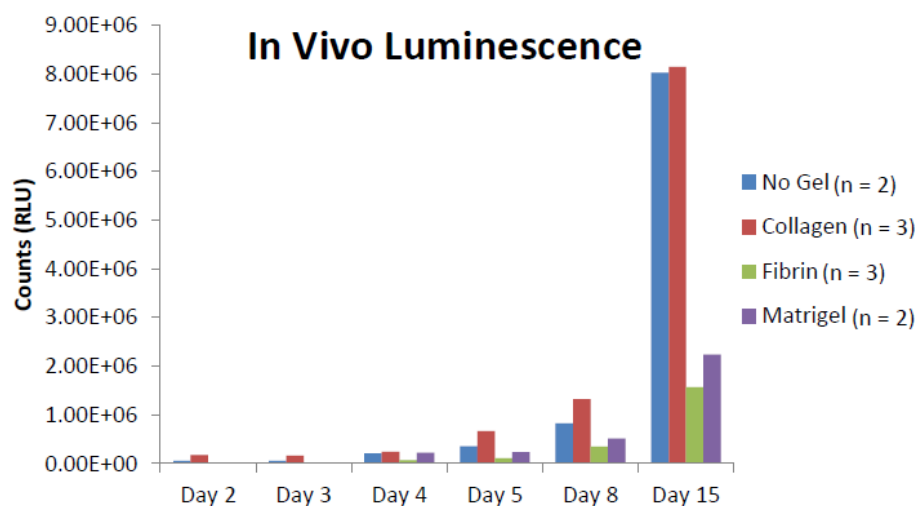


Figure 5. Quantitative analysis of Xenogen bioluminescence imaging.

In addition to quantifying cell retention at the site of injection, we will also be analyzing osteoclastogenesis of delivered RAW iRANK cells. On Day 15, mice were sacrificed and the right calf muscle was harvested and processed for histology of the ectopic bone nodules. We will perform immunohistochemistry for GFP to confirm the presence of RAW iRANK cells at the site of HO formation. Additionally, we will perform TRAP staining to determine if RAW iRANK cells form osteoclasts *in vivo* in response to CID treatment.

During the previous year we have continued to refine our HO model. Specifically, we have implemented a stereotaxic microinjection protocol that will allow us to induce HO lesions in the calf midbelly in a highly reproducible manner. The stereotaxic coordinates used for inducing HO will then be used for the subsequent cell injection, which will allow us to deliver cells with high precision to sites of HO formation.

SOW Major Task 2: Test the ability of murine engineered osteoclasts to resorb calcified deposits in human HO samples derived from traumatic bone injuries.

Subtask 1: Collection of human HO samples (PI: Sangeorzan, Harborview)

We have currently established a protocol for the collection and storage of human HO specimens. During the next year we will begin collecting human HO samples. HO samples will be fixed in 70% ethanol and stored at -80°C for future experiments to determine the ability of engineered osteoclasts to resorb calcification in human HO samples.

SOW Major Task 3: To develop human iPS cells as a cell source for engineered osteoclasts

Subtask 1: Lentiviral construct, lentiviral production, and transduction of human iPS cells.

Lentiviral construct: (PI: Giachelli, UW) Cloning of the human RANK cDNA into a CID-regulatable lentiviral vector has been completed. The human RANK cDNA was cloned into the pCDH_EF1_MCS_T2A_copGFP lentiviral vector (Figure 6). This vector contains a multiple cloning site (MCS) under control of the Elongation Factor 1 alpha (EF1a) promoter. The EF1a promoter was chosen because it has been shown to induce robust, long-term transgene expression human pluripotent stem cells and cells of the hematopoietic lineage. The lentiviral expression cassette also contains a GFP reporter gene downstream of a “self-cleaving” T2A peptide. The T2A peptide allows expression of the human RANK cDNA and the GFP reporter from the same promoter.



Figure 6. Schematic depiction of the lentiviral vector encoding a CID-regulatable human RANK. LTR = long terminal repeat; EF1a = elongation factor 1-alpha; M = myristoylation sequence; F36V = FKBP12 domain; F36V' = modified FKBP12 domain; hRANK = cytoplasmic domain of human RANK.

The cloning strategy for the CID-regulatable RANK lentivirus construct is depicted in Figure 7. The details of the cloning are as follows. The Myr-F36V'-F36V fragment was PCR amplified from the pEMlenti_GFP_IRES_Myr_F2_mRANK plasmid and XbaI and EcoRI restriction enzyme sites were introduced. Following amplification, the fragment was digested with XbaI and EcoRI and ligated into the multiple cloning site of pCDH_EF1_MCS_T2A_GFP to create pCDH_EF1_M_F2_T2A_GFP. The RANK cytoplasmic domain was PCR amplified from a human RANK cDNA template (Promega) to which EcoRI and BamHI sites were introduced. The RANK fragment was digested with EcoRI and BamHI and ligated into the vector pCDH_EF1_M_F2_T2A_GFP at the C-terminal end of the second F36V domain. The resulting pCDH_EF1_M_F2_hRANK_T2A_GFP vector was confirmed via DNA sequencing and restriction digest analysis.

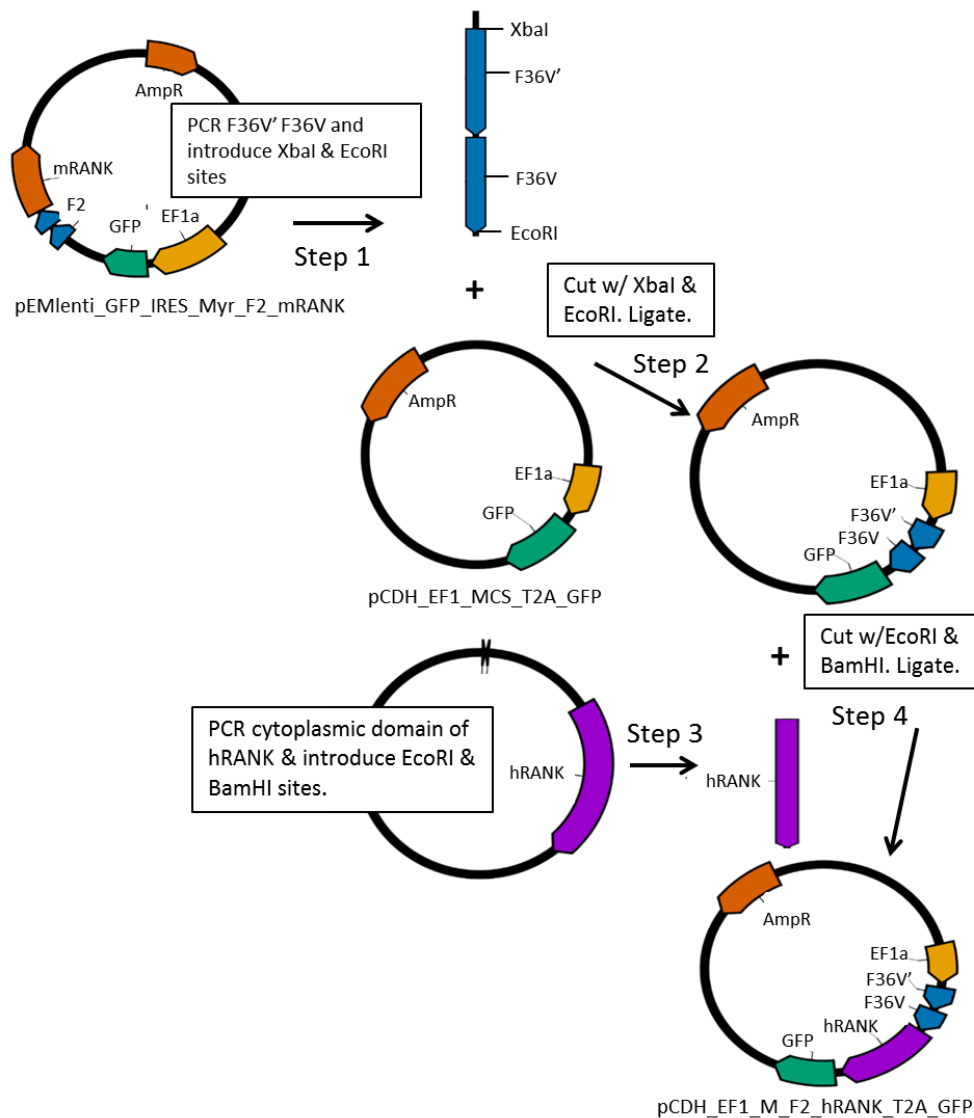


Figure 7. Cloning strategy for the human CID-regulatable lentiviral construct

Lentiviral production: (PI: Giachelli, UW) The pCDH hRANK vector was used to generate human iRANK lentivirus. The human pCDH hRANK plasmid together with the lentiviral packaging plasmids (pSL3, pSL4, and pSL5) were transfected into HEK293T packaging cells using Lipofectamine 2000. Lentivirus containing supernatant was collected at 48 and 72 hours post-transfection. Viral supernatant was concentrated via ultracentrifugation.

CID-responsiveness of human iRANK construct: (PI: Giachelli, UW) Before testing the human iRANK lentiviral construct directly in human iPS cells, we validated the vector in two cell lines. In initial studies, the HL-60 human

promyelocytic cell line was chosen because stimulation of these cells with RANK ligand induces phosphorylation of the kinase, ERK (Figure 8) and it is therefore a convenient cell line for testing CID-regulatable RANK responses.

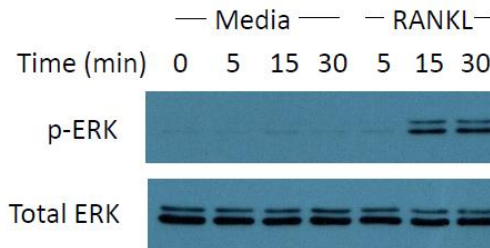


Figure 8. RANKL induces ERK phosphorylation in HL-60 cells. HL-60 cells were treated with RANKL or media alone and total ERK and phosphorylated ERK (p-ERK) were assayed by Western blot.

To test the CID-responsiveness of the human iRANK construct, HL-60 cells were transduced with the human iRANK lentivirus and sorted for expression of the GFP reporter via FACS. HL-60 hiRANK cells were plated in 12-well plates in DMEM/10% FBS and allowed to grow for 48 hours. Media was switched to DMEM/1% FBS for 24 hours. Cells were then stimulated with RANKL, AP20187, or 0.1% EtOH (vehicle control) in DMEM/1% FBS and cell lysates were collected in cell lysis buffer with protease and phosphatase inhibitors at 0, 15, 30, and 60 minutes post-stimulation. Cell lysates (10 ug) were run on SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed for total ERK and phosphorylated ERK (p-ERK). As shown in Figure 9, stimulation with AP20187 resulted in dose-dependent phosphorylation of ERK compared to cells treated with the vehicle control (EtOH).

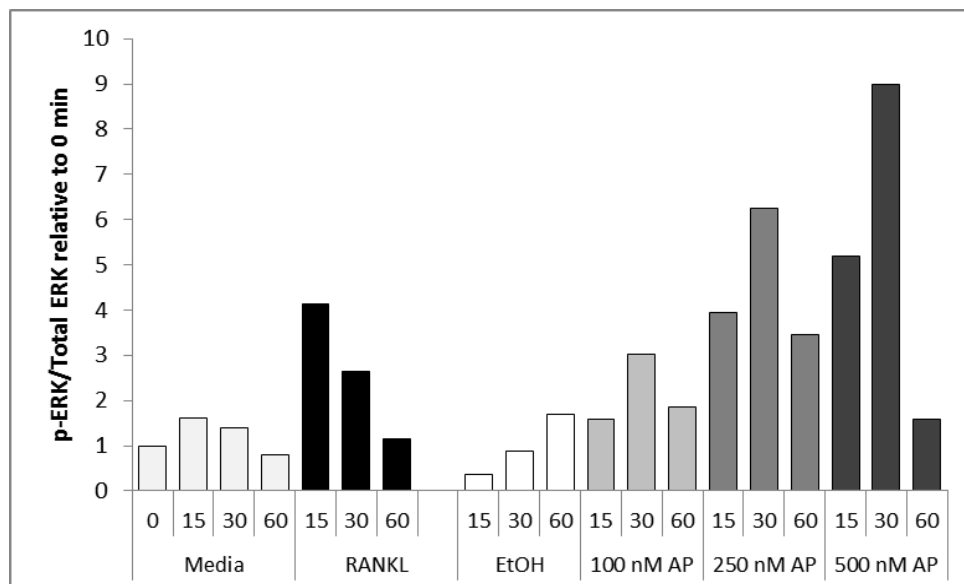


Figure 9. AP20187 induces ERK phosphorylation in HL-60 hiRANK cells. HL-60 hiRANK cells were treated with RANKL, 0.1% EtOH (vehicle control) or AP20187 and total ERK and phosphorylated ERK (p-ERK) were assayed by Western blot.

In addition to testing the human RANK lentiviral construct in HL-60 cells, we also tested the construct in RAW264.7 cells for its ability to induce osteoclast formation. RAW264.7 cells were transduced with human iRANK lentivirus and sorted for expression of the GFP reporter via FACS. RAW264.7 hiRANK cells were seeded in 12-well plates at 1.5×10^4 cells/well. Cells were treated with AP20187 or 0.1% EtOH (vehicle control) for 5 days. Cells were then fixed and stained for TRAP. As shown in Figure 10, treatment with AP20187 resulted in the formation of multinucleated TRAP-positive cells in RAW hiRANK cells.

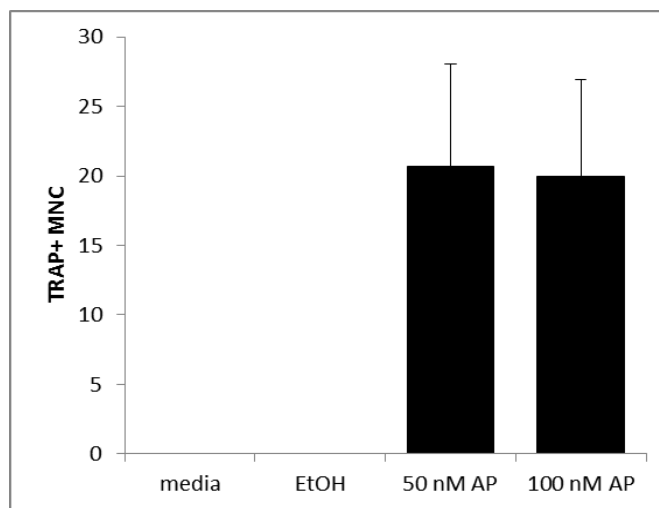


Figure 10. AP20187 induces osteoclast formation in RAW264.7 hiRANK cells. RAW264.7 hiRANK cells were treated with AP20187 or 0.1% EtOH for 5 days. Cells were then fixed and stained for TRAP.

Validation of osteoclast differentiation protocol for human iPS cells: (PI: Giachelli, UW) This year we initiated studies to test the osteoclast directed differentiation protocol published by Choi et al. using the human iPS(foreskin)-1 cell line. This differentiation protocol (Figure 11) is composed of three major steps: (1) induction of hematopoietic differentiation by co-culture of hiPSCs with OP9 bone marrow stromal cells; (2) expansion of hematopoietic progenitors with GM-CSF; and (3) directed differentiation of myeloid progenitors into osteoclasts. Choi et al, reported that 17.2×10^6 osteoclasts could be generated from one 6-well plate of undifferentiated iPS cells.

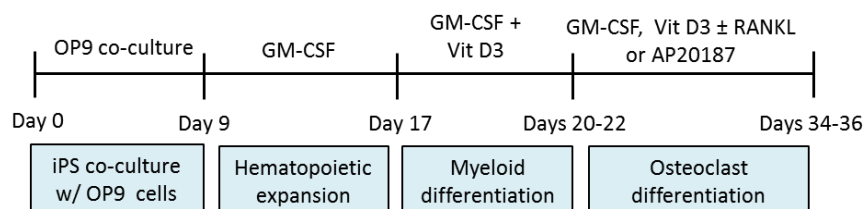


Figure 11. Directed differentiation protocol for human iPS cells.

In initial tests, we unexpectedly obtained a poor yield of hematopoietic progenitors and tested multiple lots of FBS to determine which lot supports the highest efficiency of hematopoietic differentiation. For these experiments, iPS(foreskin)-1 were plated on OP9 stromal cells to initiate hematopoietic differentiation. After 9 days of co-culture, cells were harvested with collagenase and analyzed via flow cytometry for expression of CD43, CD235a, CD41, and CD45. However, we found no significant differences in the number of CD43⁺CD235a/41⁻CD45⁺ multipotent progenitor cells generated using the different lots of FBS. One possible explanation for this is the high level of spontaneous adipogenesis we observed in the OP9 stromal cells used for the co-culture differentiation protocol (Figure 12). When OP9 cells undergo spontaneous adipogenesis they no longer support hematopoietic differentiation. Consequently, we have obtained a new lot of OP9 cells from the ATCC and are testing these for their ability to induce hematopoietic differentiation of hiPSCs. We are also testing different formulations of media (powdered vs. liquid), FBS (characterized vs. defined), and ascorbic acid concentration to determine the optimal parameters for retaining the OP9 in the undifferentiated state.

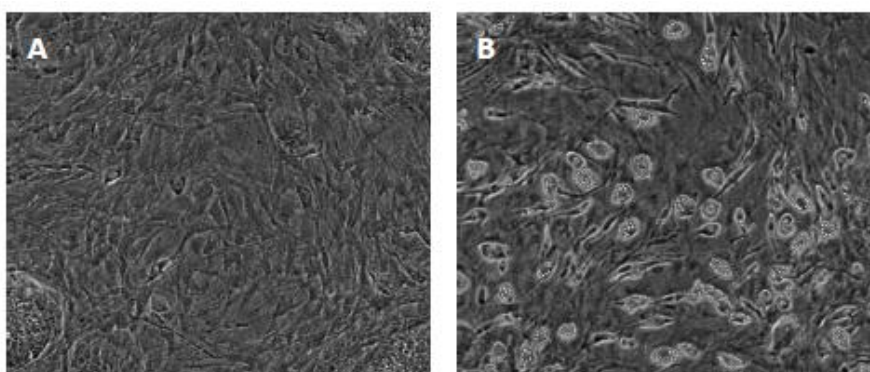


Figure 12. Morphological changes in OP9 stromal cell cultures. (A) Confluent monolayer of OP9 cells for co-culture with hiPSCs. (B) Spontaneous adipogenesis in OP9 cells.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Created a lentiviral vector containing a CID-inducible human RANK fusion gene and a green fluorescent protein reporter gene. (PI: Giachelli, UW)
- Verified CID-responsiveness of the human iRANK lentiviral construct in HL-60 and RAW264.7 cells (PI: Giachelli, UW)
- Developed a method for encapsulating engineered RAW iRANK cells in collagen, Matrigel, and fibrin hydrogels that allows for efficient generation of osteoclasts in response to CID treatment *in vitro*. (PI: Giachelli, UW)
- Confirmed HO formation in nude mice. (PI: Sangeorzan, Harborview)
- Validated hydrogel carriers as cell delivery vehicles to retain engineered RAW iRANK cells at sites of injection. (PI: Sangeorzan, Harborview)

5. CONCLUSION:

Currently, few effective treatments exist to treat heterotopic ossification. Our studies will advance the field by determining whether osteoclasts can prevent and/or regress HO and provide insights into optimal conditions for the treatment of HO. The goals of this research are to develop proof-of-principle data in excised human HO samples and in an animal model using existing murine engineered osteoclasts and to develop an off-the-shelf human iPS cell source for clinical translation of this technology.

Future Plans

SOW Major Task 1:

- Subtask 2: Optimization of local cell delivery at time of surgical implantation and after HO has formed in mice: During the next quarter we will continue to optimize cell-delivery vehicles with the goal of retaining the engineered cells at intramuscular sites of HO formation. Further, we will determine the average number of cells retained at the injection site for each dose and gel formulation, the dose and/or gel dependent parameters that effect intramuscular localization and retention, and the repeatability of each treatment. We anticipate that the optimization of the cell-delivery will continue the entire quarter.
- Subtask 3: Optimization of CID dosing and route of administration to maximize osteoclast formation *in vivo*: After determining the optimal cell delivery vehicle to retain engineered cells at the site of HO formation we will optimize the CID dose and route of administration for osteoclast formation *in vivo*.
- Subtask 4: Local cell delivery of engineered osteoclasts to prevent and treat HO formation: After determining the optimal parameters for cell delivery and

CID administration we will proceed to efficacy studies to determine if engineered osteoclasts can prevent HO *in vivo*.

SOW Major Task 2:

- Collection of human HO samples: We have currently established a protocol for the collection and storage of human HO samples. During the next year we will begin collecting human HO specimens from patients with traumatic bone injuries. We anticipate collecting approximately 15 samples in the next year.

SOW Major Task 3:

- Optimization of osteoclast differentiation protocol for human iPS cells: We are currently evaluating multiple parameters to optimize the iPS hematopoietic differentiation protocol. These experiments are ongoing and during the next quarter we will continue optimizing the osteoclast directed differentiation protocol.

- Transduction of human iPS cells: Optimal parameters for lentiviral transduction will be determined by altering viral concentration, iPS cell density, and polybrene concentration. Transduced iPS cells will be selected via GFP expression and expanded clonally. A bank of transduced iPS cells will be created for future experiments.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Presentations:

Rementer C, Buranaphatthana W, Giachelli CM. *Characterizing an Inducible Osteoclast System as Cell Therapy for the Treatment of Ectopic Calcification*. Biomaterials Day April 2014. Poster presentation. First place prize for the Biomaterials Day poster session.

Giachelli C. *Vascular Calcification: New Concepts in Regulation and Engineering Treatments*. Bioengineering Departmental Seminar, Northwestern University. May 15, 2014

Giachelli C. *Vascular Calcification: New Concepts in Regulation and Therapy* International Vascular Biology Meeting, Kyoto, Japan, April 15, 2014

Giachelli C and Sangeorzan B. *Engineered osteoclasts for the treatment and prevention of heterotopic ossification*. Presentation to Congressman Adam Smith (D-WA), Harborview Medical Center, Seattle WA, August 4, 2014.

7. INVENTIONS, PATENTS AND LICENSES:

None to report.

8. REPORTABLE OUTCOMES:

- Created a lentiviral vector containing a CID-inducible human RANK fusion gene and a green fluorescent protein reporter gene. (PI: Giachelli, UW)
- Verified CID-responsiveness of the human iRANK lentiviral construct in HL-60 and RAW264.7 cells. (PI: Giachelli, UW)
- Developed a method for encapsulating engineered RAW iRANK cells in collagen, Matrigel, and fibrin hydrogels that allows for efficient generation of osteoclasts in response to CID treatment *in vitro*. (PI: Giachelli, UW)
- Validated hydrogel carriers as cell delivery vehicles to retain engineered RAW iRANK cells at sites of injection. (PI: Sangeorzan, Harborview)

9. OTHER ACHIEVEMENTS:

None to report.

10. REFERENCES:

N/A

11. APPENDICES:

n/a